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Low Power Laser Irradiation Reduces Ischemic Damage in Hippocampal Slices In Vitro

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Background and Objective: **Low power laser irradiation has been reported to reduce injury, promote regeneration, and produce analgesia. While the mechanism is unknown, one hypothesis is that light produces free radicals, which have a beneficial effect at low concentrations.**
Study Design/Materials and Methods: **We have investigated the effects of low power laser irradiation on the loss of electrical excitability of hippocampal brain slices after a transient exposure to a perfusion medium lacking oxygen and containing reduced glucose concentrations. Injury in this system is known to result at least in part from free radical production.**
Results: **Low power laser irradiation increased the time required for loss of excitability and increased recovery from the ischemic injury.**
Conclusions: **Low power laser irradiation has acute protective effects against ischemic damage in brain slices.**

Key words: brain slice, excitability, free radicals, hypoglycemia

INTRODUCTION

A variety of beneficial effects of low power laser irradiation have been reported [see reviews 1,2]. While several authors have questioned whether various of these reported effects are real [1,3], an increasing body of evidence supports the proposition that low power laser irradiation can promote wound healing and nerve regeneration, and may provide analgesia. These effects are believed to be independent of temperature changes, and at least some are wavelength specific.

One of the first reports of facilitated wound healing was by Mester et al. [4], who reported that both mechanical and burn wounds healed more quickly after being irradiated with a ruby red laser at 693 nm at doses of 1.0, 4.0, and 5.0 J/cm^2. Other studies [5] implicate stimulation of collagen formation in this effect. In a more systematic study, Rochkind et al. [6] demonstrated statistically significant increases in recovery from cutaneous skin flap and burn wounds with a 632.8 nm laser applied for 7 min each day for 21 days at 7.6 J/cm^2. These investigators also studied effects on nerve injuries. While there was no effect of irradiation on sciatic nerve compound action potentials, they report that irradiation significantly increased the rate and degree of recovery of the compound action potential after a nerve crush injury. The irradiation was accompanied by a decreased degeneration of motor neurons to the affected muscles.

Various other authors have reported effects on nerve regeneration consistent with the conclusions of Rochkind et al. [6]. Anders et al. [7] demonstrated a statistically significant increase of re-

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generation of the facial nerve after unilateral crush and daily irradiation at 633 nm, 8.5 mW for 90 min. Irradiation with the same protocol but at 361, 457, 514, 720, or 1064 nm did not have effect. A similar wavelength dependency was also found in an independent study by Rochkind et al. [8] using crushed sciatic nerve, in which the authors report a reduction in the loss of functional nerve activity in the irradiated animals. These observations were extended by Rosner et al. [9], who reported that irradiations at 632.8 nm begun immediately before injury were as effective as those immediately after injury. There was no effect of irradiation at 904 nm. Basford et al. [10], however, applied irradiation at 830 nm to evaluate median nerve function in a double-blinded study in human volunteers. They found significant decreases in motor and sensory nerve latencies distal to the irradiation site. Others have reported elevations of brain serotonin levels in irradiated animals relative to controls [11], and a decrease in synaptic density in hippocampus following irradiation of rat pups from 1–5 days of age [12]; however, neither of these studies has been replicated. Other authors have reported that analgesia is produced by irradiation at 780 nm [13] or 830 nm [14], and provide some evidence that these actions are mediated by suppression of nociceptive afferent fiber activity.

We have examined the effects of low energy laser irradiation on a different injury model system, namely, ischemic damage to the isolated perfused hippocampal brain slice. These isolated slices are stable and functional for many hours if provided sufficient oxygen and glucose. However, when the slice is perfused with a medium containing reduced glucose concentration and lacking oxygen, one can obtain a graded loss of electrical excitability with time, which is reversible provided that glucose and oxygen are restored quickly [15,16]. We have used this injury model to determine whether there is a protective effect of low power laser irradiation, and we find that such actions do exist.

MATERIALS AND METHODS

Rats (200–250 g) were euthanized by cervical dislocation under light ether anesthesia, brains quickly removed and transferred into ice-cold Krebs-Ringer solution, containing 126 mM NaCl, 5 mM KCl, 1.26 mM KH_2PO_4, 1.3 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM glucose. The brains were blocked, and slices (450 μm thickness) were cut perpendicular to the long axis of the hippocampus using the Nomiyama vibratome. Slices were incubated in Krebs-Ringer solution saturated with 95% O_2, 5% CO_2 at 34°C for 2 hours before being removed to the recording chamber. The slices were submerged (about 1 mm below the fluid level) on a plexi-mesh and perfused at a rate of about 3 ml/min at 34°C. When it was desired to subject the slice to ischemic conditions, a modified Krebs-Ringer was perfused, containing only 2.5 mM glucose and equilibrated with 95% N_2, 5% CO_2.

Figure 1 is a schematic view of the preparation, showing the slice, the stimulating and recording electrodes, and the laser. A monopolar stimulating electrode was placed in the Schaffer collateral pathway in order to synaptically activate the pyramidal neurons in the CA1 area of hippocampus. Stimulation pulses were 50 μs in duration, about 15 V in intensity and were applied at an interval of 5 s. Population field potentials were recorded in the CA1 pyramidal cell layer with a glass electrode (tip diameter 10–20 μm) filled with Krebs-Ringer solution. The light guide of the low power soft laser (a helium-neon laser of wavelength 632.8 nm with a power of 6 mW continuous wave, Model Soft Laser 632, manufactured by World Wide Laser Industry, SA Geneva) was positioned just over the recording site for irradiation as described in a previous report [17].

Figure 2 shows the experimental protocol. Each slice was placed in the recording chamber and the experiment begun after a stable population response was obtained. Data were collected and stored via an on-line computer using data analysis software (Data Translation DT2801 and DT208A) and presented using Lotus 1-2-3. Five minutes of control responses were collected, followed by a 5 min perfusion of the oxygen-free, low glucose Krebs-Ringer, and then returned to control Krebs-Ringer for a 15 min recovery period. This schedule was followed in the absence (trace A) or the presence (trace B) of He-Ne laser irradiation for the full 25 min experimental period. After irradiation each slice was retested as in the control (trace C) to determine long-lasting effects of the irradiation, if any.

RESULTS

Stimulation of the Schaffer collateral pathway induces monosynaptic excitation of the pyramidal neurons in CA1, and this synaptic response

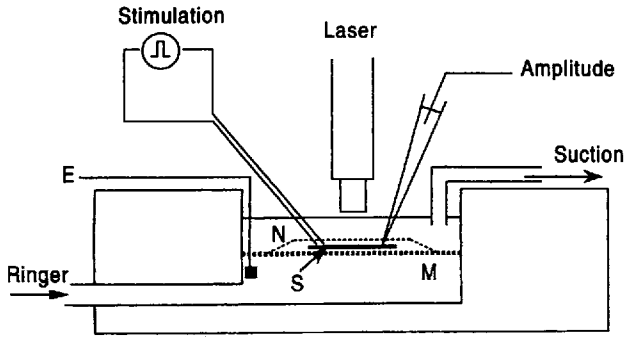

Fig. 1. Schematic diagram of the recording and exposure system. The hippocampal slice (S) was positioned under a nylon net (N) on top of a plexiglass mesh (M) and submerged in flowing Krebs-Ringer solution which was removed from the top of the chamber through a tube by suction. The recording was done using a glass micro electrode which was positioned in the cell body layer of CA1, and the signal against a bath reference electrode (E) recorded through an amplifier. The Schaffer collateral pathway was activated via a stimulator and a bipolar electrode positioned in the pathway. The light guide of the laser was positioned directly over the submerged slice.

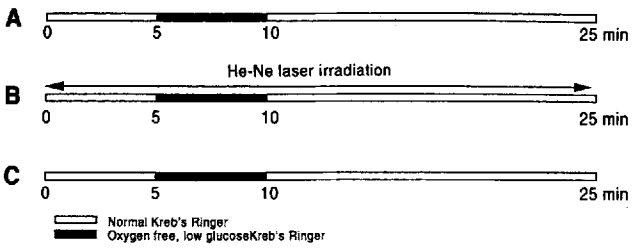

Fig. 2. Sequence of experiments. Open bars indicate perfusion of normal oxygenated Krebs-Ringer, while solid bars indicate perfusion of oxygen-free, low glucose Krebs-Ringer. For all three types of exposure, peak negative-to-positive monosynaptic population responses were elicited and recorded each 5 sec. After stability of recording was achieved, data were collected for five minutes (0 to 5) under control conditions, then slices perfused with the ischemic medium for 5 min (5 to 10), followed by wash with normal Krebs-Ringer for 15 min (10 to 25). Control conditions (without irradiation) are indicated in **A**, while **B** shows slices exposed to the He-Ne laser for the full 25 min period of the experiment. Slices exposed as in B were then given a repeated ischemic trial after the wash in B, but without continued irradiation. This is shown as **C**.

was recorded as an early biphasic population response as shown in Figure 3. Later waveforms are sometimes seen, reflecting polysynaptic events, but these events were not studied in detail. Figure 3B shows the characteristic control response, recorded at minute 3 (during the control period), minute 7 (after ischemia for 2 min), minute 11 (after return to control Krebs-Ringer for 1 min),

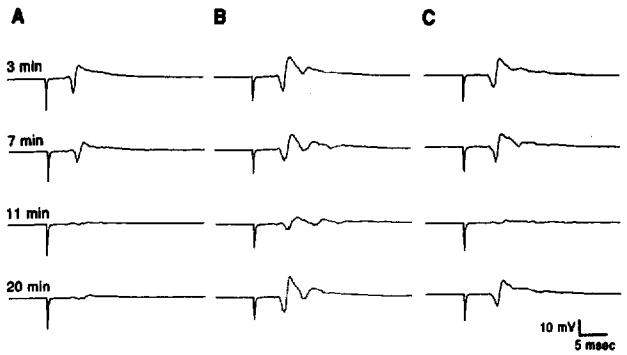

Fig. 3. Representative raw data records of population responses from slices under control conditions (**A**), during irradiation (**B**), and upon exposure to a second ischemic challenge without irradiation but after irradiation on the first test (**C**). The records illustrated correspond to control (3 min), 2 min of ischemia (7 min), 1 min wash (11 min), and 10 min wash (20 min).

and minute 20 (after 10 min wash). The postsynaptic responses were reduced and ultimately totally blocked by perfusion of the oxygen-free, low glucose Krebs-Ringer solution, and under control conditions showed little or no return of excitability after 10 min wash.

Application of low power laser irradiation had no consistent effect on the amplitude of the population responses. However, such irradiation did alter the response to ischemia, as shown in Figure 3B. With irradiation the degree of decline of the population response during the standard 5 min ischemic period was reduced. There was a rapid recovery to control after the 5 min ischemic period in the presence of irradiation.

The effects of irradiation appear to be relatively long lasting, since when a slice first tested with low power laser irradiation was subsequently re-tested without irradiation, there was a much greater degree of recovery than seen in slices that had not been previously irradiated. Figure 3C shows this experiment from the same slice studied in Figure 3B, where the sequence of ischemia exposure was repeated just after termination of the laser irradiation. Under these circumstances, there was almost total loss of excitability during the ischemic period, but unlike controls there was a nearly complete recovery after a 10 min wash with normal Krebs-Ringer.

Figure 4 shows plots of the peak negative-to-positive response amplitude for the three experiments illustrated in Figure 3. This plot illustrates how markedly laser irradiation serves to reduce the degree of loss of excitability, and how rapidly

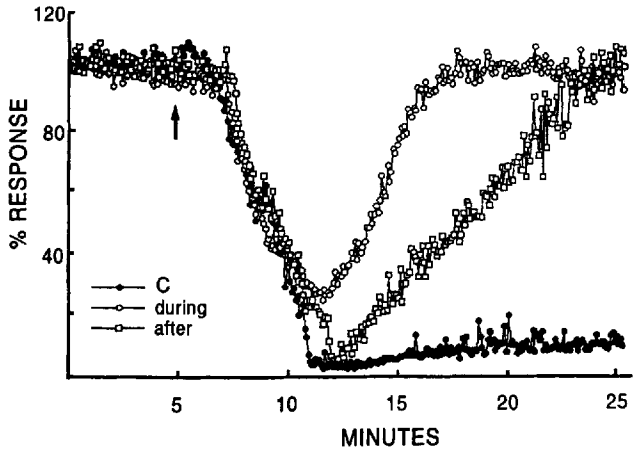

Fig. 4. Plot of the computer analyzed peak amplitude for the complete experiments illustrated in Figure 3, expressed as percentage of control response amplitude. Control responses are indicated by closed circles, responses during the laser exposure are open circles, and responses obtained from the same slice, but in a second trial without laser exposure, are indicated as open squares. The ischemic exposure occurred between minutes 5 and 10, and the onset is indicated by the arrow.

the response recovers from the ischemic insult. Note that the time course of recovery of the previously irradiated slice (open squares) is considerably slower than seen during the irradiation, but ultimately in this slice recovery to control values was achieved. Since there was essentially no recovery in the 15 min post-ischemic period in the control slice, this observation indicates that there is some residual beneficial effect of the irradiation.

Table 1 summarizes the data from the 16 experiments performed, where each slice was characterized by the rate and degree of recovery after the 5 min ischemic period. Slices were categorized as showing no recovery if the population response at the end of the 15 min wash period was less than 10% of control, and were included in the complete recovery column if the response had returned to 100 ± 10% of the control value at the end of the wash. All other slices were reported as showing partial recovery. The different distribution of recovery between the controls and during laser irradiation is significant of the 0.001 level by the chi-square test for independence.

Conceivably this effect of low power laser irradiation on ischemic damage is secondary to local heating, although at these power levels one would not expect much heating. In order to determine whether a laser-induced temperature rise could explain these results, we investigated the effects of temperature on the loss of excitability following perfusion of oxygen-free, low glucose Krebs-Ringer solution. These results are shown in Figure 5 where the solution change occurred at the arrow. As indicated in this figure, the loss of excitability was more rapid at 34°C than at 30°C. This is, in fact, the expected result, since metabolic activity is greater at higher temperatures. Since the effect of temperature is opposite that which would be required to explain the effects of low laser irradiation on the loss of excitability in the studies shown in Figures 3 and 4, we conclude that the low laser irradiation is not activity secondary to a change in temperature.

TABLE 1. Recovery After Ischemia as a Function of Laser Irradiation

	Degree of recovery		
	None	Partial	Complete
Control slices	4	2	0
Laser-exposed slices	0	1	4
Slices made ischemic after irradiation	0	5	0

DISCUSSION

We have demonstrated a consistent effect of low power laser irradiation in reducing a quite different form of cellular injury than has previously been studied. When nervous tissue is acutely deprived of both oxygen and glucose, a variety of events occur that relatively quickly result in loss of electrical excitability. Glucose is the exclusive source of metabolic energy in neurons, and all metabolism requires oxygen. Ischemia of this form results in a rapid fall in intracellular ATP [18], release of excitatory neurotransmitters [19] and inhibitory agents like adenosine [20], and a total failure of the process of synaptic transmission [15]. These events are all reversible provided that the ischemia is not prolonged.

It is somewhat surprising to find that low power laser irradiation alters these acute processes. Most previous reports of effects of low power laser irradiation occur much more slowly, as is the case with wound healing or nerve regeneration. One of the few reports of a rapid beneficial action is that of Rochkind et al. [8], who demonstrated that He-Ne laser irradiation applied directly to a bare nerve in vivo reduced the decrement of action potentials when the nerve was

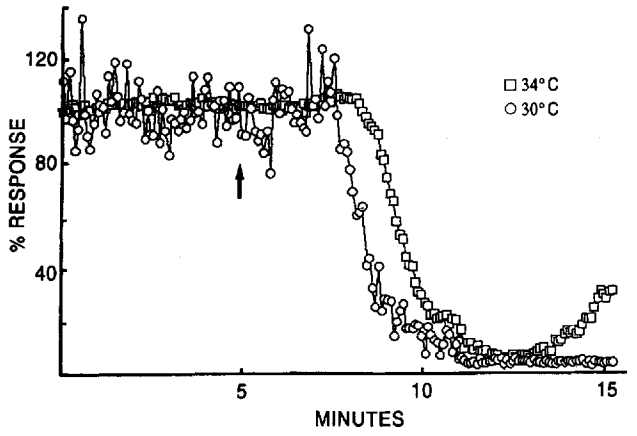

Fig. 5. Effects of temperature on time course of loss of excitability following ischemia and recovery after return to control. Data compared from an experiment at 34°C (open circles) and one at 30°C (open squares). The onset of ischemia is indicated by the arrow at 5 min.

subsequently crushed. This effect was apparent immediately after crush, and lasted for 1 day.

The mechanisms whereby low power laser irradiation exerts these beneficial effects are unknown, although several investigators have made speculations. Olson [21] has suggested that the primary event is absorption of light by mitochondrial enzymes, while Karu [22] has suggested that the absorption of light by the respiratory chain components, such as the flavins and cytochromes, results in an acceleration of electron transfer in parts of the respiratory chain. Lubart et al. [23] have reported that singlet oxygen is produced by low power laser irradiation, and propose that this may be a common mechanisms of action. Exactly how singlet oxygen is produced is not totally clear, but Lubart et al. [23] and Rochkind and Ouaknine [2] suggest that it is secondary to the interaction of light with chromophores (probably porphyrins) in the cells. What is less clear is how singlet oxygen production could have a beneficial effect, since it is well known that production of free radicals is destructive in most tissues [24–26]. Ischemic damage both in brain and in brain slices has been shown to be associated with free radical production, and is reduced by agents which reduce production or scavenge free radicals [27,28]. Thus it appears very unlikely that in this system there could be any beneficial effect of free radical production, given the strong evidence that a major factor in the loss of excitability is the excessive free radical production produced by the lack of oxygen and glucose. Nevertheless, the beneficial effect of low power laser irradiation is clear, and must result from some other presently unknown mechanism.

Iwasa et al. [17] have reported low power laser actions with intracellular recordings from neurons in brain slices, and found restorative actions on mildly damaged neurons, but no obvious effects on neurons with a normal and stable membrane potential. They report no effects on very severely damaged neurons.

In conclusion, our demonstration of a protective action of low power laser irradiation of the loss of excitability of a mammalian brain slice exposed to lack of oxygen and glucose is clear, but the mechanisms responsible are not. Much further study of the mechanisms whereby low power lasers exert these potentially important actions is needed.

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REFERENCES

1. Basford JR. Low-energy laser treatment of pain and wounds: Hype, hope, or hokum? Mayo Clin Proc 1986; 61:671–675.
2. Rochkind S, Quaknine GE. New trend in neuroscience: Low-power laser effect on peripheral and central nervous system (basic science, preclinical and clinical studies). Neurol Res 1992; 14:2–11.
3. Jongsma FHM, Bogard NJC, Hulsberg JP. Is closure of open skin wounds in rats accelerated by argon laser exposure? Lasers Surg Med 1983; 3:75–80.
4. Mester E, Spiry T, Szende B, Tota JG. Effect of laser rays on wound healing. Am J Surg 1971; 122:532–535.
5. Kana JS, Hutschenreiter G, Haina D, Waidelich W. Effect of low-power density laser radiation on healing of open skin wounds in rats. Arch Surg 1981; 116:293–296.
6. Rochkind S, Rousso M, Nissan M, Villarreal M, Barr-Neal L, Rees DG. Systemic effects of low-power laser irradiation on the peripheral and central nervous system, cutaneous wounds, and burns. Lasers Surg Med 1989a; 9:174–182.
7. Anders JJ, Borke RC, Woolery SK, Van De Merwe WP. Low power laser irradiation alters the rate of regeneration of the rat facial nerve. Lasers Surg Med 1993; 13: 72–82.
8. Rochkind S, Nisson M, Lubart A. A single transcutaneous light irradiation to injured peripheral nerve: Comparative study with five different wavelengths. Lasers Med Sci 1989b; 4:259–263.
9. Rosner M, Caplan M, Cohen S, Duvdevani R, Solomon A, Assia E, Belkin M, Schwartz M. Dose and temporal parameters in delaying injured optic nerve degeneration by low-energy laser irradiation. Lasers Surg Med 1993; 13: 611–617.

10. Basford JR, Hallman HO, Matsumoto JY, Moyer SK, Buss JM, Baxter GD. Effects of 830 nm continuous wave laser diode irradiation on median nerve function in normal subjects. Lasers Surg Med 1993; 13:597–604.
11. Cassone MC, Lombard A, Rossetti V, Urciuoli R, Rolfo PM. Effect of *in vivo* He-Ne laser irradiation on biogenic amine levels in rat brain. J Photochem Photobiol B: Biol 1993; 18:291–294.
12. Igarashi H, Inomata K. Effects of low-power gallium aluminum arsenide diode laser irradiation on the development of synapses in the rat neonatal rat hippocampus. Acta Anat 1991; 140:150–155.
13. Honmura A, Ishii A, Yanase M, Obata J, Haruki E. Analgesic effect of Ga-Al-As diode laser irradiation on hyperalgesia in carrageenin-induced inflammation. Lasers Surg Med 1993; 13:463–469.
14. Sato T, Kawatani M, Takeshige C, Matsumoto I. Ga-Al-As laser irradiation inhibits neuronal activity associated with inflammation. Acupuncture Electro-Therapeutics Res Int 1994; 19:141–151.
15. Hori N, Doi N, Miyahara S, Shinoda Y, Carpenter DO. Appearance of NMDA receptors triggered by anoxia independent of voltage *in vivo* and *in vitro*. Exp Neurol 1991; 112:304–311.
16. Hori N, Carpenter DO. Functional and morphological changes induced by *in vivo* ischemia. Exp Neurol 1994; 129:1–5.
17. Iwase T, Hori N, Morioka T. Possible mechanisms of the He-Ne laser effects on the cell membrane characteristics. *Laser Med Surg* 1988; 4:166–171.
18. Kass I, Lipton P. Mechanisms involved in irreversible anoxic damage to the *in vitro* rat hippocampal slice. J Physiol Lond 1982; 332:459–472.
19. Rothman SM, Olney JW. Glutamate and the pathophysiology of hypoxic/ischemic brain damage. Ann Neurol 1986; 19:05–111.
20. Van Wylen DL, Park TS, Rubio R, Berne RM. Increases in cerebral interstitial fluid adenosine concentration during hypoxia, local potassium infusion and ischemia. Cereb Blood Flow Metab 1986; 6:522–528.
21. Olson J, Schimmerling W, Tobias CA. Laser action spectrum of reduced excitability in nerve cells. Brain Res 1981; 204:436–440.
22. Karu TI. Molecular mechanism of therapeutic effect of low intensity laser irradiation. Laser Life Sci 1988; 2:53–74.
23. Lubart R, Malik Z, Rochkind S, Fisher T. A possible mechanism of low level laser-living cell interaction. Laser Ther 1990; 2:65–68.
24. Floyd RA. Role of oxygen free radicals in carcinogenesis and brain ischemia FASEB J 1990; 4:2587–2597.
25. Ames BN, Shigenaga MK, Hagen TM. Oxidants, antioxidants, and the degenerative diseases of aging. Proc Natl Acad Sci USA 1993; 90:7915–7922.
26. Levine RL. Ischemia: From acidosis to oxidation. FASEB J 1993; 7:1242–1246.
27. Flamm ES, Desmopoulos HB, Seligman ML. Free radicals in cerebral ischemia. Stroke 1978; 9:445–453.
28. Kitagawa K, Matsumoto M, Oda T, Ninobe M, Hata R, Handa N, Fukunaga R, Isaka Y, Kimura K, Maeda H, Mikoshiba K, Kamada T. Free radical generation during brief period of cerebral ischemia may trigger delayed neuronal death. Neuroscience 1990; 35:551–558.